Attorney's Dock t No. 5051-425 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Dominique Robertson
Serial No.: 09/281,528
Filed: March 30, 1999
For: METHOD OF SUPPRESSSING GENE EXPRESSION IN PLANTS

Group Art Unit: 1633
Examiner: S. Kaushal

Commissioner for Patents
Washington, DC 20231

Declaration of Dominique Robertson, Ph.D.

Pursuant to 37 C.F.R. § 1.132

I, Dominique Robertson, do hereby declare and say as follows:

1. I am a named inventor on United States Application No. 09/281,528 ("the '528 application") and of the subject matter claimed therein.

2. I have a Ph.D. degree in Plant Cell Biology from Cornell University in Ithaca, NY. I am an Associate Professor of Botany and Genetics in the Department of Botany at North Carolina State University. I have been conducting research in the area of geminivirus induced gene silencing for 10 years and have co-authored 3 publications related to the area of geminivirus-induced gene silencing and 6 publications related to the area of geminivirus-host interactions.

3. The investigations described below were carried out in my laboratory at North Carolina State University in Raleigh, North Carolina, USA, under my direction and supervision according to the protocols set forth in the '528 application. These studies demonstrate that the geminivirus silencing vector and DNA constructs that are the subject matter of the composition and method claims pending in the '528 application can comprise a variety of geminivirus genomes and a variety of heterologous DNA sequences comprising at least a fragment of a plant gene endogenous to a variety of plants. On the basis of these studies, it is my belief that the claims of the present invention could be practiced with any geminivirus genome, any heterologous DNA sequence and any plant.

4. **Geminivirus genomes studied**

Attached herewith as Exhibit A are xcerpts from manuscripts and slides showing data produced from studies in which geminivirus silencing vectors as claimed in the present invention and employing a geminivirus genome of tomato

golden mosaic virus and cabbag leaf curl virus were produced and tested for their ability to silence expression of an endogenous plant gen upon introduction into a plant cell. A.1. shows the structure of TGMV-derived silencing vectors and the location and size of tested heterologous DNA fragments from a cDNA encoding a gene required for chlorophyll formation. A.2. demonstrates that slight variations in sequence between the target host gene and the heterologous DNA carried by the silencing vector can still elicit a strong silencing response. A.3. shows that a second, distantly related geminivirus with a conserved genome organization can also be used as a silencing vector. A.4. compares silencing of homologous genes, the tobacco Su (sulfur) gene encoding a magnesium chelatase subunit and the *Arabidopsis* CH42 (chlorata 42) gene. The TGMV *su* construct produces extensive silencing in *N. benthamiana* and the CbLCV *ch42* construct produces extensive loss of chlorophyll in *Arabidopsis*. A.5. shows a table comparing sequence similarity of geminiviruses and demonstrates that the TGMV and CbLCV are distantly related and that several economically important viruses, such as Sida Golden Mosaic Virus, which infects cotton, are intermediate between tomato golden mosaic virus and cabbage leaf curl virus.

5. **Heterologous DNA sequences studied**

Attached herewith as Exhibit B are excerpts from published and unpublished manuscripts showing data produced from studies in which geminivirus silencing vectors as claimed in the present invention and employing a variety of heterologous DNA sequences were produced and tested for their ability to silence expression of an endogenous plant gene upon introduction into a plant cell. B.1. is a summary of TGMV vectors that have been inoculated into *Nicotiana benthamiana*. Some genes upregulated by geminivirus infection were silenced to determine if they were necessary for viral infection. These included PCNA and 3 unknown genes, clone 9, 25, and 37, (B.2.) identified by PCR-based subtraction of infected and uninfected plants (Eagle and Robertson, unpublished). Homologs of these genes were tested in *Arabidopsis* to determine if they were needed for CbLCV infection (B.4.). Also tested in *Arabidopsis* were genes identified in other labs as being necessary for silencing (B.4.). There is precedent in *C. elegans* for silencing a gene that is known to be required for silencing. These experiments verified results in *Arabidopsis* null mutants, that RdRp (host RNA dependent RNA polymerase) was required for geminivirus induced gene silencing (chapter 5, Muangsan Ph.D. thesis). The retinoblastoma related protein and proliferating cell nuclear antigen proteins (B.2.) are essential for plant growth and would be embryo lethals if mutated. B.3. is a summary of CbLCV vectors that have been inoculated into *Arabidopsis*.

6. **Plant species studied**

Attached herewith as part of Exhibit A are excerpts from manuscripts and slides showing data produced from studies in which geminivirus silencing vectors as claimed in the present invention and employing a variety of heterologous DNA sequences were produced and tested for their ability to silence expression of an endogenous plant gene upon introduction into the cells of plants of different species. Figures 2 and 3 in section A.4. show that different plants (*Nicotiana*, a member of the

Solanaceae family and *Arabidopsis*, a member of the Brassicaceae family) infected with different geminivirus vectors show a similar response, uniform loss of chlorophyll in new growth, when homologous, endogenous genes are targeted.

These data demonstrate that two different geminiviruses, 17 different heterologous DNA sequences and species from two different plant families were successfully employed in the compositions and methods of the claimed invention.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dominique Robertson, Ph.D.

11/25/02
Date

Exhibit A

A. 1 T mato G lden Mosaic Virus Vectors, as des ribed in Peele et al. (2001) Plant J. 27: 357

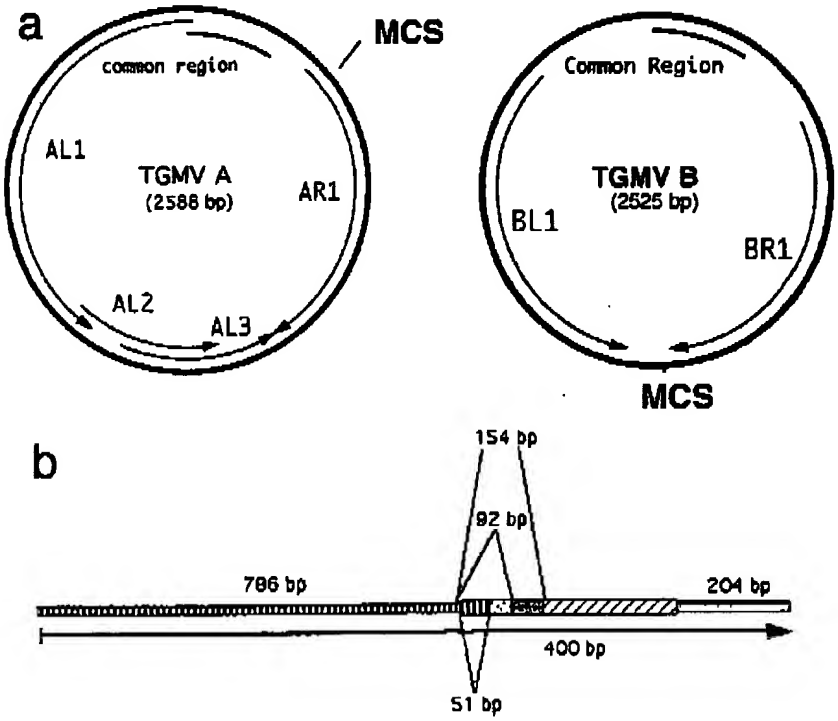


Figure 1. TGMV A- and B-derived episomal silencing vectors.
(a) Silencing DNA fragments homologous to endogenous gene(s) are inserted into a multiple cloning site (MCS). In the A component vector, silencing fragments are transcribed from the AR1 promoter and are inserted in place of the AR1 gene. In the B component vector, silencing DNA is inserted 20 bp downstream of the BR1 stop codon, and is cotranscribed with BR1. The viral genes, AL1, AL2, AL3, BL1 and BR1, are needed for replication and movement of the vector. The common region is identical in the two components and contains the origin of replication.
(b) Location of gene fragments from the su cDNA used for silencing. The arrow shows the su cDNA and the ATG and TAA mark the beginning and end of the gene.

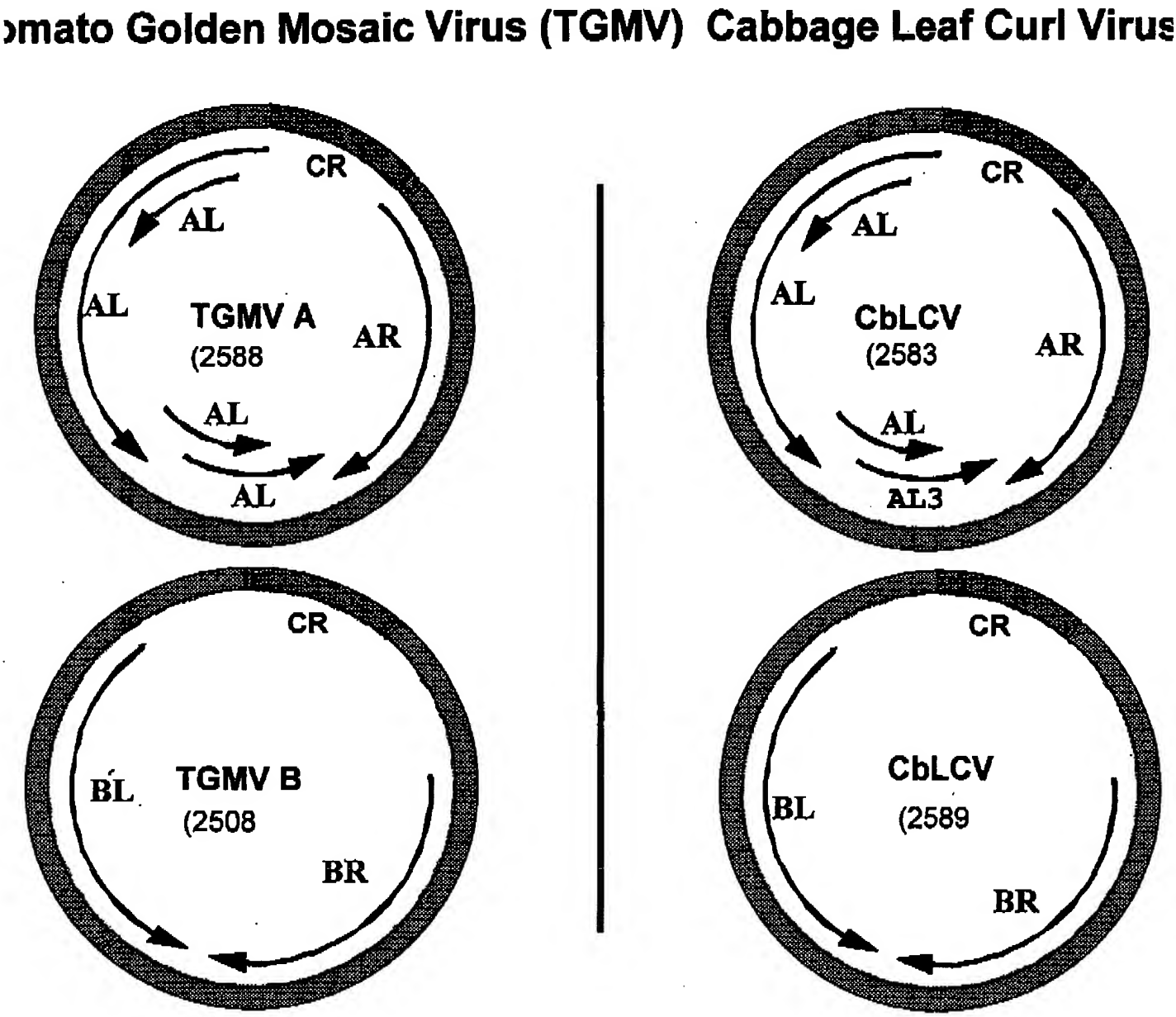
The TGMV A vector is a replacement vector. Heterologous DNA replaces the AR1 gene and is transcribed using the AR1 promoter. The TGMV B is an insertional vector. Heterologous DNA is inserted into the MCS \ and is cotranscribed with the BR1 gene. The size limitation is approximately 160 bp. This work proves that geminiviruses with

monopartite genomes, having no dispensable genes such as AR1, can be engineered as insertional vectors for gene silencing.

A.2. Consensus homology between N. benthamiana (endogenous gene) and N. tabacum (silencing fragment) for magnesium chelatase. In Figure 1b, section A.1., a Nicotiana tabacum cDNA sequence for a gene encoding one of three subunits of the enzyme magnesium chelatase (required for chlorophyll formation) is shown. Below is a comparison of the sequences in N. benthamiana (target gene for silencing) and N. tabacum (heterologous sequences cloned into TGMV to initiate silencing in N. Benthamiana). Fragments of the N. tabacum cDNA between 91 and 795 bp produced reliable silencing. This demonstrates that 100% homology between the target and the viral vector insert (heterlogous DNA) is not absolutely required for silencing to occur. N indicates a non-conserved nucleotide.

ATGGCTTCACTnnTnGGnACTTCCTCTTCAGCAGCAGCTGCTGCAATATT
AGCTTCTACACCCTTnTCTTCTCGCTCCTnTAAnnCTnCCnTTTTCTCCC
TCTTCCCTTCTTCAGGGCAGnGTCAAGGGAGGAAGTTTTATGGAGGGATT
AGAnTCCCAGTTAAGAAAGGGAGGTCCCAATTnCATGTGGCAATTTCAAA
TGTTGCnACGGAAnTCAACCCTGCTCAAGAACAGGGTCAGAAACTTGCTG
AGGAGAGCCAGAGACCnGTGTATCCATTTGCAGCTATAGTGGGACAAGAn
GAnATGAAGTTATGTCTTTTGCTGAATGTAATTGATCCAAAGATTGGAGG
TGTGATGATAATGGGTGATnGnGGnACCGGGAAGTCCACCACGGTTAGAT
CTTTGGTAGATTTACTTCCTGAnATCAAAGTTATTTCTGGTGATCCGTTC
AATTCAGATCCAGATGACCAAGAAGTAATGAGTGCAGAAGTCCGTGACAA
ATTGAGGAGCGGAnAGnAGCTTCCTATATCTCGTACCAAAATCAACATGG
TTGATTTACCGCTAGGTGCTACTGAnGACAGGGTGTGTGGCACAATCGAC
ATTGAGAAAGCTCTTACTGAGGGTGTGAAGGCTTTCGAnCCTGGTCTTCT
TGCTAAAGCTAACAGAGGAATnCTTTATGTCGATGAnGTTAATCTTTTGG
AnGACCATTTAGTAnATGTTCTTTTGGATTCTGCAGCATCGGGATGGAAC
ACTGTTGAAAGAnAnGGGATATCAATnTCACAnCCnGCCCGATTTATCCT
TATTGGTTCnGGTAATCCTGAAnAAnGAGAACTTAnGCCACAACTTCTTG
ATCGATTTGGAATGCATGCCCAAGTGGGGACCGTGAGAGATGCAGAGCTG
AGAGTGAAGATCGTTGAGGAAAGAGCTCGTTTTGATAAGAACCCCAAGGA
ATTCCGnGAGTCATACAAGGCAGAGCAAGAAAAGCTCCAGAATCAAATCG
ACTCAGCTAGGAACGCTCTTTCTGCTGTTACAATnGATCATGATCTTCGA
GTTAAAAnCTCTAAGGTCTGTGCAGAACTnAAnGTCGATGGATTGAGAGG
TGATATAGTCACTAACAGGGCAGCAnGAGCGTTGGCTGCACTAAAAGGAA
GAGATAAGGTnACTCCGGAnGATATCGCCACTGTCATTCCCAACTGCTTA
AGACACAGnCTnAGnAAnGAnCCnT

A.3. Comparison of cabbage leaf curl virus and tomato golden mosaic virus genomes. The genus begomovirus has a conserved genome structure.



A.4. Tw different viruses carrying DNA fragments that target homologous g nes (coding for a subunit f magn sium chelatase) required for chlorophyll f rmation ause effective llencing and loss of chlorophyll in both N. benthamiana and Arabidopsis.

Fig. 2. TGMV carrying a 154 bp DNA fragment (SU) inoculated into N. benthamiana.

Fig. 3. CbLCV carrying a 400 bp DNA fragment (CH42) inoculated into Arabidopsis.

A.5. Genome con rvation in members of the Geminivirldii, g nus b gom virus.

Table 1 lists the amino acid similarity of selected geminiviruses and demonstrates that cabbage leaf curl virus, with only 69% amino acid identity with tomato golden mosaic virus, silences as effectively as tomato golden mosaic virus. Table from Turnage et al. (2002) The Plant J. 30:107. Work in another lab (Atkinson et al. 1998 The Plant J. 15:593) demonstrates the monopartite geminiviruses of the genus mastrevirus (tobacco yellow dwarf virus) can also be engineered for silencing vectors. They developed an insertional vector for silencing chalcone synthase in Petunia.

able 1. Amino acid similarity of selected new world (NW) and old world (OW) begomoviruses

irus	Average[a]	AL1/AC1[a]	BL1/BC1[a]
abbage leaf curl virus	**100 (100)**	**100 (100)**	**100 (100)**
ean calico mosaic virus	76 (86)	84 (90)	75 (87)
quash leaf curl virus	74 (84)	81 (88)	75 (86)
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omato leaf curl virus	71 (81)	65 (75)	81 (89)
omato leaf crumple virus	71 (83)	65 (78)	80 (89)
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butilon mosaic virus	69 (81)	59 (73)	81 (89)
omato golden mosaic virus	**69 (81)**	**60 (74)**	**78 (88)**
omato rugose mosaic virus	68 (80)	60 (74)	76 (86)
epper huasteco virus	67 (80)	52 (68)	82 (91)
avana tomato virus	66 (78)	62 (75)	70 (79)
assava latent virus	44 (60)	53 (68)	44 (61)
dian cassava mosaic virus	42 (60)	51 (68)	43 (58)
igna mungo yellow mosaic virus	42 (61)	52 (68)	44 (63)
outh African cassava mosaic virus	41 (58)	51 (67)	42 (59)
lungbean yellow mosaic virus	41 (59)	51 (67)	43 (61)
laterm lon chlorotic stunt virus	41 (59)	51 (68)	43 (58)
lest African cassava mosaic virus	40 (57)	51 (67)	41 (56)
dian mungbean yellow mosaic virus	40 (60)	51 (67)	44 (62)
omato yellow leaf curl virus	39 (59)	53 (68)	39 (59)

B

Exhibit B Summary of genes that have been silenced using geminiviruses
B.1. Nicotiana benthamiana inoculated with TGMV vectors carrying different inserts.

Construct	Heterologous fragment	Target gene	Silencing phenotype
pSK16L	Luciferase, 650 bp	Accession no. P08659	Loss of transgenic luciferase luminescence (Kjemtup et al., 1998)
pCPTGMV::GFP	Green fluorescent protein, 780 bp	Accession no. AAB47998	Loss of GFP fluorescence (Peele et al., 2001)
pCPTGMV A::su	786 bp *Acc*651/*Eco*RV fragment, corresponding to nt 0-786 of su cDNA, antisense orientation	Accession no. AAG35472	Loss of chlorophyll (Kjemtrup et al., 1998; Peele et al., 2001)
pCP1.3BPCNA	Proliferating cell nuclear antigen, 122 bp	Accession No. AF486816	Loss of primary plant growth (Peele et al., 2001)
pNMTGMV B::Clone 9	106 bp *Ssp*I/*Eco*RV fragment, corresponding to 96 bp 5' cDNA of the Clone 9 961 bp fragment upregulated by viral replication	Unpublished, homology to (*A. thaliana*) NP563717.1	Loss of viral symptoms in new growth (Muangsan, Ph.D. thesis 2002)
pNMTGMV B::Clone 37	Unknown protein (clone 37) upregulated by viral replication protein	Unpublished, homology to (*A. thaliana*) AAD27878	Loss of viral symptoms and DNA in new growth (Eagle, Muangsan and Robertson, unpublished)
pNMTGMV B::Clone 8	Unknown proteins (clone 8) upregulated by viral replication	Unpublished	Loss of viral symptoms in new growth (Eagle, Muangsan and Robertson, unpublished)
pNMTGMV B::Clone 25.1	Clone 25.1, with homology to calmodulin-related protein	Unpublished, homology to (*A. thaliana*) NP198593	Loss of viral symptoms in new growth (Eagle, Muangsan and Robertson, unpublished)
pCJTGMV::Rb	150 bp fragment of tobacco Retinoblastoma related protein	BAA76477	Severe necrosis in inoculated leaf, lesions in upper leaves, attenuated symptoms, flowers curl (Jordan and Robertson, unpublished)

B.2. Phenotypic changes reflecting downregulation of target genes by TGMV silencing vectors

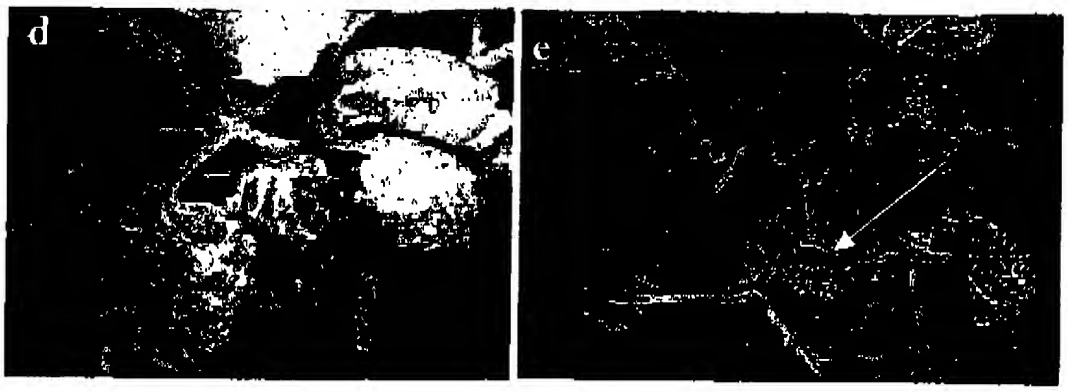


Figure 1. Attenuated symptoms in *Nicotiana benthamiana* plants silenced in *clone 9*. Plants were inoculated with a combination of TGMV A and TGMV B::SU (d) or TGMV A and TGMV B::Clone 9 (e) and photographed four weeks post inoculation. Arrow (e) show symptoms in lower leaves that were eliminated in new growth.

Figure 2. N. benthamiana silenced with TGMV carrying a DNA fragment from clone 37. Mild symptoms are shown (arrow) in newly infected tissue but upper growth shows no sign of symptoms and lacks viral DNA.

Fig. 3 N. benthamiana inoculated with TGMV vector containing a fragment of the retinoblastoma gene. Left, cell death in vegetative growth and attenuated viral symptoms. Right, flower curling 360°. Normally, flowers are long and straight.

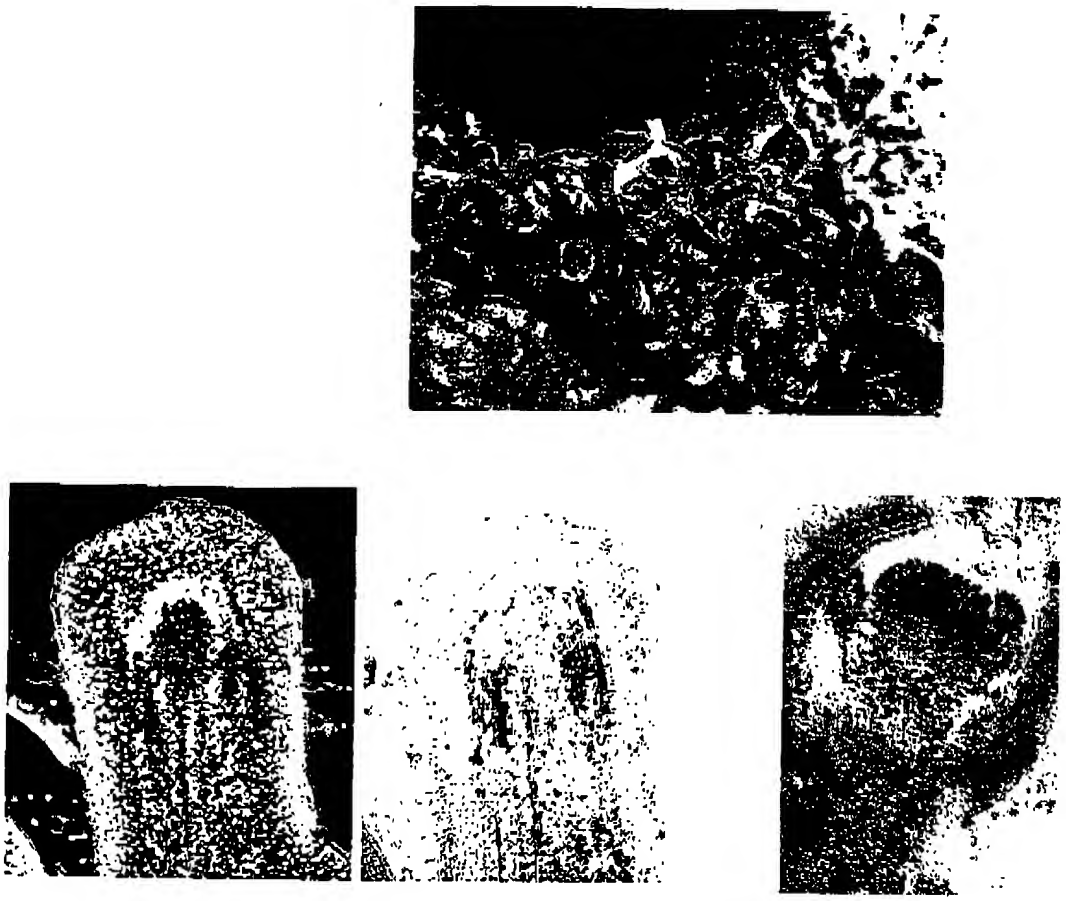


Fig. 4 N. benthamiana silenced for PCNA. Top – loss of apical growth. Bottom left, immunolocalization of PCNA in silenced meristems (center) demonstrates loss of PCNA expression compared to wt (right). Left shows the same meristems as center visualized for nuclei using DAPI.

B.3. Cabbage Leaf Curl Vectors inoculated into Arabidopsis

CbLCV construct	Heterologous DNA	gene	phenotype
pMTCbLCVA::CH42A	360 bp of homology to the *ch42* gene, antisense orientation	accession no. P16127	Loss of chlorohyll (Turnage et al., 2002)
pNMCbLCVA::CH42S	406 bp fragment with 374 bp of homology to the *ch42* gene, sense orientation	accession no. P16127	Loss of chlorohyll (Turnage et al., 2002)
pNMCbLCVA::luc	618 bp of homology to the *luciferase* gene, sense orientation	Accession no. P08659	Control, no homology to endogenous gene
pNMCbLCVA::PCNA	412 bp fragment, corresponding to nt 115-526 of *PCNA1* cDNA, antisense	accession No. NM100611	Reduced viral accumulation
pNMCbLCVA::CaMRP	400 bp fragment, corresponding to nt 21-420 of *CaMRP* cDNA, antisense	accession No. AY117325	No detectable phenotype
pNMCbLCVA::AtG1RP	398 bp fragment, corresponding to nt 1026 to 1421 of *AtG1RP* cDNA, antisense	accession No. AY054498	No detectable phenotype
pNMCbLCVA::SGS2	434 bp fragment, corresponding to nt 2211-2644 of *SGS2* cDNA, antisense	Accession No. AF239718	Reduces CH42 silencing (light green vs white)
pNMCbLCVA::SDE3	437 bp fragment, corresponding to nt 1591-2027 of *SDE3* cDNA, antisense	accession no. AF339908	CH42 silencing is uneven
pMTCbLCVA::PDS	370 bp fragment from phytoene desaturase	Accession no. AAA20109	Loss of chlorophyll and carotenoids (Turnage et al., 2002)
pMTCbLCVA::GFP	388 bp fragment from mGFP5	Accession no. AAB47998	Loss of green fluorescence in GFP transgenic plants (Turnage et al., 2002)
pNMCbLCVB::CH42	144 bp *Bam*HI/*Eco*RV fragment of the	accession no. P16127	Loss of chlorohyll (Turnage et al.,

	ch42 cDNA, antisense orientation		2002)
pNMCbLCVB::CH42	144 bp *Bam*HI/*Eco*RV fragment of the *ch42* cDNA, sense	accession no. P16127	Loss of chlorohyll (Turnage et al., 2002)

B.4. Selected examples of silencing phenotypes in Arabidopsis inoculated with CbLCV vectors carrying different inserts

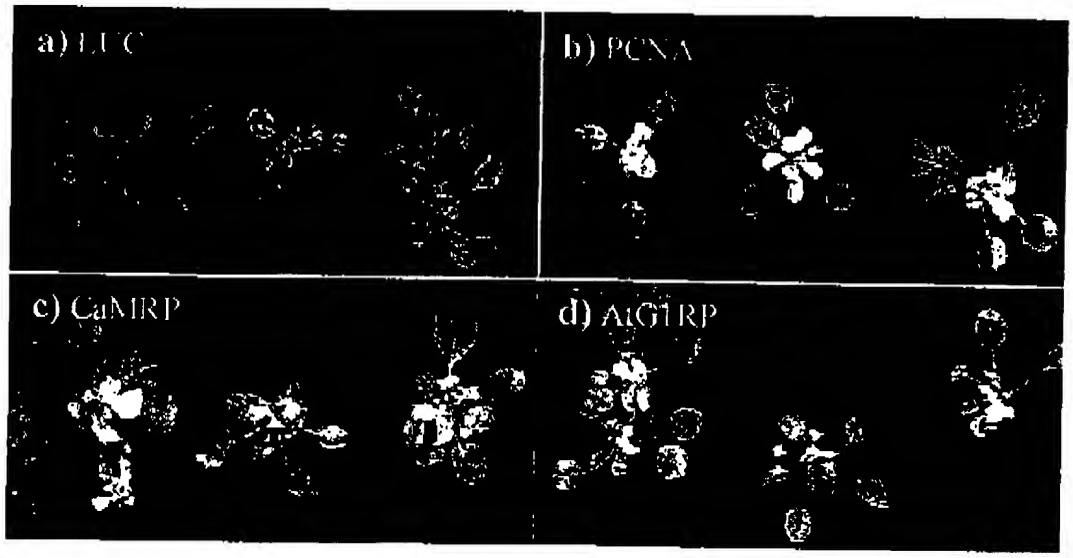
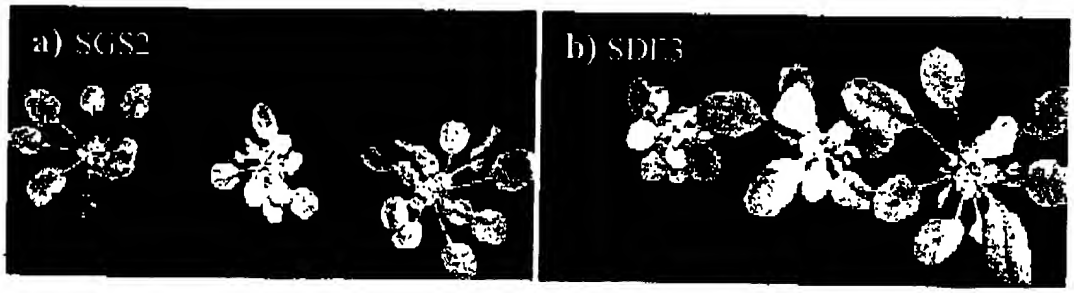


Figure 7. VIGS of *PCNA*, *CaMRP*, and *AtG1RP* endogenous genes.
Wild type Arabidopsis plants four-weeks-old were inoculated with CbLCV::luc or CbLCV carrying a fusion of *CH42* fragment and one of these three genes: *PCNA*, *CaMRP*, or *AtG1RP*. CH42 silencing, yellow tissue, were observed in PCNA (b), CaMRP (c) and AtG1RP (d) plants. Photographs were taken at 25 dpi.



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